Plant Cell Physiol. 35(5): 811–820 (1994)
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Identification and Characterization of Acidic Hydrolases with Chitinase and Chitosanase Activities from Sweet Orange Callus Tissue[1]

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Acidic chitinases (EC 3.2.1.14) were isolated and characterized from 4-week-old nonembryogenic *Citrus sinensis* L. Osbeck cv 'Valencia' callus tissue. The enzymes were purified using size exclusion, anion exchange, and chromatofocusing HPLC techniques. Eleven isoforms were isolated with M_rs between 26,000 and 37,400. Eight of the isoforms were purified to homogeneity, and all but one cross-reacted with a polyclonal antibody raised against a basic class I potato leaf chitinase. The isoelectric points (determined by chromatofocusing) were from pH 4.5 to 5.4. All hydrolases degraded chitin and four were capable of hydrolyzing solubilized shrimp shell chitosan suggesting they may be chitosanases (EC 3.2.1.99). Apparent chitosanase activity generally decreased with decreasing acetylation of the chitosan (i.e. from 20% to 0% acetylation). The chitinases and chitinases/chitosanases are predominantly endochitinases. Chitosanase activity was optimal at pH 5 while the pH optimum for chitinase activity ranged between pH 3.5 and 5.5. The chitinases and chitinases/chitosanases were stable up to 60°C and showed their highest enzyme activity at that temperature. N-terminal sequences were obtained on three of the isoforms. One of the isoforms was identified as a class II chitinase and the other two as class III chitinases.

Key words: Citrus — Defensive proteins — Hydrolases — Lysozyme — Plant tissue culture — Pathogenesis-related proteins.

Plants possess many means of defense against pathogens, most of which are passive. An incompatible, resistant host-pathogen interaction is characterized by specific hypersensitive defense reactions induced in the host after the pathogen is recognized. These active defense mechanisms include hypersensitive responses (e.g., necrosis), synthesis of inhibitory compounds such as phytoalexins (Ebel 1984), and PR proteins (Carr and Klessig 1989). PR proteins are induced in monocots and dicots as a result of plant-pathogen interactions. These proteins are well known in tobacco plants infected with TMV and are divided into several families for classification purposes (Bol et al. 1990). PR protein families 2 and 3 have been identified as β-1,3-glucanases (EC 3.2.1.39) and chitinases (EC 3.2.1.14), respectively. The hydrolases in PR protein families 2 and 3 are widely distributed among plants, and their activities have been shown to increase in bean, pea, tomato, cucumber, barley, and maize leaves infected with different pathogens (Carr and Klessig 1989, Bol et al. 1990, Flach et al. 1992).

Investigations by Fink et al. (1988), Martin (1991), and

Abbreviations: A0, 1, 2, etc., anion exchange column fractions; AS, ammonium sulfate; BSA, bovine serum albumin; CF1, 2, 3, etc., chromatofocusing column fractions; GlcN, glucosamine; GlcNAc, *N*-acetylglucosamine; HPLC, high performance liquid chromatograph; PAGE, polyacrylamide gel electrophoresis; PR, pathogenesis-related; SEC, size exclusion chromatography; SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography; TMV, tobacco mosaic virus.

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Collada et al. (1992) showed that some plant chitinases are also constitutive enzymes. Chitinases and β-1,3-glucanases are inducible in several plants by treatment with ethylene or ozone (Boller et al. 1983, Mauch and Staehelin 1989, Schraudner et al. 1992, Koga et al. 1992), but induction by such elicitors is not a criterion for being a PR protein.

Not all proteins that qualify as PR proteins have recognized families. For example, Grenier and Asselin (1990) described some PR proteins which showed lytic activity against fungal spores and acted only on chitosan and not on chitin; these were identified as chitosanases (EC 3.2.1.99). Chitosanases from different bacteria and fungi were first described by Monaghan et al. (1973). Recently, Ouakfaoui and Asselin (1992a, b) reported on chitosanases between 10,000 and 23,000 M_r in different tissues of several monocots and dicots.

It is known that chitinases can degrade fungal cell walls either alone (Schlumbaum et al. 1986) or in concert with β-1,3-glucanases (Mauch et al. 1988b). There have been a few reports indicating that some chitinases also possess chitosanase activity (Pegg and Young 1982, Osswald et al. 1993). Osswald et al. (1993) have suggested that enzymes possessing both chitinase and chitosanase activities would be advantageous to plant defensive mechanisms as a wider variety of plant pests could be attacked. It may well be that all three enzymes (i.e., β-1,3-glucanases, chitinases, and chitosanases) work in concert against plant pests.

Our laboratory recently initiated studies on chitinases and chitosanases in citrus (Osswald 1992, 1993). Our interest has been in the possibility of using these enzymes either separately or in conjunction with other PR and PR-like proteins to reduce production and postharvest losses due to insects and pathogens. In this paper we describe the purification and characterization of several acidic chitinases and chitinase/chitosanases from nonembryogenic sweet orange callus tissue.

Material and Methods

Chemicals—Shrimp shell chitosan (18% acetylated as determined by IR spectroscopy; Osswald et al. 1992) routinely used for chitosanase assays was purchased from Atomergics Chemetals Corp. (Farmingdale, NY; Lot LO729). Chitosans used for substrate specificity and endo- and exohydrolytic determinations were as described in Osswald et al. (1992) except for a crab chitosan that was 5% acetylated (No. 4C-5) which was a gift of Dr. Aiba (National Institute of Chemical Research, Ibaraki, Japan). Fluorescamine (Fluram) was obtained from Fluka (Buchs, Switzerland). The low molecular weight markers (M_r 14,500, 21,500, 31,000, 45,000, 66,000, and 97,000) were from BioRad (Richmond, CA), and the Polybuffer 74 from Pharmacia (Piscataway, NJ). BSA (fraction V), 5-bromo-4-chloro-3-indolyl phosphate, and nitro blue tetrazolium were purchased from Sigma (St. Louis, MO) and U.S. Biochemicals (Cleveland, OH), respectively.

Citrus tissue culture—A nonembryogenic cell line (Val 88-1) was developed from immature fruit vesicles of *Citrus sinensis* (L.) Osbeck cultivar 'Valencia' as reported recently (Osswald et al. 1992, 1993).

Preparation of 100% deacetylated chitosan—Two fully deacetylated chitosan samples were used. Completely deacetylated crab shell chitosan was a gift from Katakura Chikkarin Co., Ltd. (Ibaraki, Japan). Shrimp shell chitosan (Atomergics Chemetals Corp.) was completely deacetylated as described by Domard and Rinaudo (1983).

Chitinase assay—Chitinase activity was measured as previously reported (Osswald et al. 1993) using colloidal [^{3}H]chitin from shrimp shell chitosan. The specific activity of the prepared [^{3}H]chitin (605 μCi per mol GlcNAc) was determined after acid hydrolysis (Mayer et al. 1980) via fluorometric analysis (Chen and Mayer 1981). Reaction time and temperature were 30 min and 37°C, respectively, unless specified otherwise. One unit of chitinase activity is defined as the release of 1 nmol GlcNAc per min.

Chitosanase assay—The chitosanase assay followed Osswald et al. (1992, 1993) using solubilized shrimp shell chitosan. Reactions were conducted at 37°C for 45 min unless specified otherwise. One unit of chitosanase activity is defined as the release of 1 nmol GlcN per min.

Immunoblotting—Proteins were separated using SDS-PAGE (14% gels) with 2-mercaptoethanol according to Laemmli (1970) and blotted onto a Immobilon-P transfer membrane (PVDF, pore size 0.45 μm, No. IPVH 304FO, Millipore, Bedford, MA) using a semidry electroblotting system according to the manufacturer's instructions (Pharmacia). Blotting was carried out as described by Osswald et al. (1993). The primary antibody was raised in rabbits against a basic (class I) potato leaf chitinase and was obtained from Dr. E. Kombrink (Max Planck Institute, Cologne, Germany). Information on the cross-reactivity and preparation of the antibody has been reported (Witte 1991).

Enzyme extraction and purification—All purification steps are shown in Figure 1. Callus tissue (2.8 kg) was harvested 4 weeks after transfer and extracted following the procedure of Osswald et al. (1993). Briefly, the tissue was powdered in liquid nitrogen, extracted with 5 liters 100 mM acetate buffer, pH 5 for 30 min, and then centrifuged for 15 min at 20,000 × g. The combined supernatants were subjected to 0% to 40%, 40% to 60%, and 60% to 90% AS precipitation for 1 h. The AS fractions were centrifuged for 15 min at 15,000 × g, and the pellets were resuspended separately in ca. 400 ml of 20 mM phosphate buffer, pH 6.7. The pellet obtained from the 40% to 60% AS fractionation showed the highest protein, chitinase, and chitosanase levels and was therefore used for all further purification steps. The resuspended AS pellets were de-

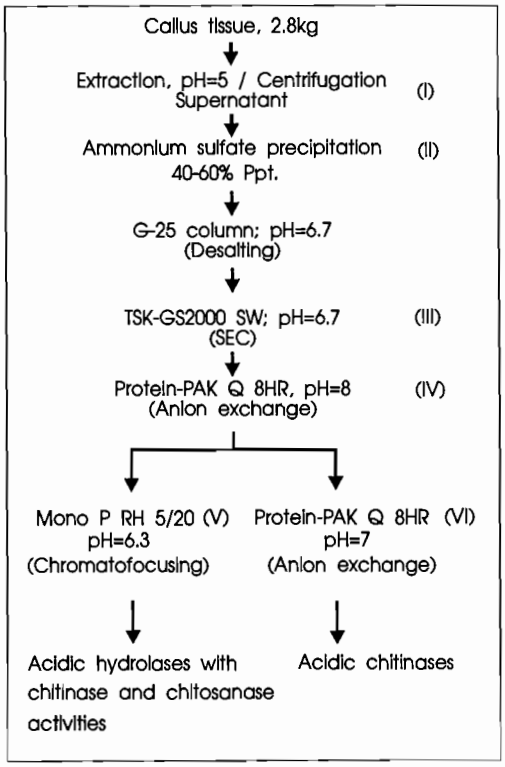

Fig. 1 Purification scheme for chitinases and chitosanases from sweet orange callus tissue.

salted on G-25 columns (5 × 25 cm) equilibrated with 20 mM phosphate buffer, pH 6.7. Active fractions were combined and concentrated using Centriprep-10 concentrators (Amicon, Danvers, MA). Subsequently, aliquots (100 μl, 1.2 mg protein) of the desalted AS fractions were separated on an HPLC SEC column (TSK-gel GS2000SW, TosoHaas, Montgomeryville, PA) equilibrated with 100 mM phosphate buffer, pH 6.7, containing 0.1 M Na_2SO_4. The HPLC system was as described previously (Osswald et al. 1993). SEC fractions containing chitinase and chitosanase activities were combined (the activities eluted identically), dialyzed for 5 h against 30 mM Tris buffer (pH 8), concentrated with Centriprep-10 concentrators and aliquots (800 μl, 1.5 mg protein) of the pooled SEC fractions were subjected to anion exchange column chromatography. The anion exchange column (Protein-Pak Q 8HR, 10 × 100 mm, Waters, Milford, MA) was equilibrated with buffer A (20 mM Tris buffer, pH 8) and elution (1 ml per min) of the proteins was accomplished using a combination of buffers A, B (0.1 M sodium phosphate, pH 6.7, 0.1 M Na_2SO_4), and C (20 mM Tris, pH 8, 0.5 M NaCl). Active fractions were identified, pooled (where peaks were determined) and concentrated with Centricon-10 concentrators. In some cases fractions (A2, A3, and A5) were further purified on a chromatofocusing column (Mono P RH 5/20, Pharmacia) with a linear gradient starting with 25 mM bis-Tris, pH 6.3 and ending with Polybuffer 74 (1 : 10 dilution) either at pH 4.7 (A2 and A3) or pH 3.3 (A5); flow rate was 1 ml per min. Fraction A4 was purified by subjecting it to a second anion exchange column run. The column was equilibrated with buffer D (20 mM bis-Tris, pH 7) and the proteins were eluted using a combination of buffers D and E (20 mM bis-Tris, pH 7, 0.5 M NaCl) at a flow rate of 1 ml per min.

Protein staining—Protein staining was done using an advanced silver staining kit from BioRad (Silver Stain Plus, BioRad No. 161-0449) according to the manufacturer's instructions.

N-Terminal sequencing—Proteins were blotted from SDS-PAGE minigels onto Immobilon P^{SQ} transfer membranes. Membranes were washed in water, stained in 0.02% Coomassie blue/40% methanol/5% acetic acid, and destained in 40% methanol/5% acetic acid for 1 min or less. Stained protein bands were excised on membrane segments for sequencing on ABI model 470 and 473A protein sequencers. Sequencing was performed at the Center for Biotechnology, University of Florida, Gainesville, FL.

Chitinase/chitosanase activity characterization—The pooled SEC fractions were used to characterize chitinase and chitosanase activity. The effects of pH were measured within the pH range of 2 to 9.5. Citrate buffer (0.2 M) was used from pH 2 to 6.5 and 0.2 M Tris buffer was used from pH 7 to 9.5. Optimal reaction temperature was determined over a 10 to 90°C range. The effects of temperature on enzyme stability were determined by preincubating the reaction mixture without substrate at various temperatures for 30 min. At the end of this time the reaction mixtures were allowed to equilibrate at 37°C; after temperature equilibration substrate was added to initiate the reaction. For all of these tests N=6. The amount of protein per reaction was 15 μg.

Chitosanase substrate specificity was measured in regard to the chitosan source and the percentage acetylation. Reaction times were 90 min, protein was 20 μg, temperature was 45°C, and the amount of chitosan was 0.4 mg per reaction.

Determination of endo- and exohydrolytic activities—Tests to determine endo- and exochitinase and chitosanase activities were conducted using the pooled SEC fractions. Purified crab chitin (Sigma) and completely deacetylated shrimp chitosan (described above) were used. Oligosaccharide standards (GlcNAc and GlcN, mono- to hexasaccharide) were gifts of the Katakura Chikkarin Co., Ltd. Generally the reactions were run as described using ca. 100 μg protein and 200 μg substrate in final volume of 300 μl. The reactions were conducted at 45°C for 12–14 h. Chitinase reactions were terminated by boiling for 10 min

and subsequently centrifuging (10,000 × *g*, 10 min). The supernatants were removed and diluted 1 : 3 with H_2O. Aliquots were spotted on silica gel 60 TLC plates (Merck, 0.25 mm). Chitosanase reactions were terminated by addition of 50 µl 1 M KOH then centrifuging as before. The supernatants were removed and filtered using Centricon 3 concentrators. The filtrates were freeze-dried, resuspended in 50 µl H_2O and aliquots spotted on TLC plates. The TLC plates were developed with 70/30/1 *n*-propanol : H_2O : NH_4OH in tanks with wicks (Mauch et al. 1988a). Chitin products were visualized employing *N*-(1-naphthyl)ethylenediamine (Bounias 1980) while chitosan products were visualized using fluorescamine (Chen and Mayer 1981).

Lysozyme activity—Lysozyme activity of the SEC preparation was determined following the method provided by Sigma Chemical Co. Briefly this method uses *Micrococcus lysodeikticus* (Sigma) suspensions (0.28 mg bacteria ml^{-1} 66 mM phosphate buffer) as the substrate. Assays were conducted at pH 5 and 6.24. The reaction (2 ml) was conducted in a cuvette (1 cm dia.) contained in a thermostatted cuvette holder (25°C) of a spectrophotometer. The reaction was initiated by addition of enzyme and the decrease in turbidity at $A_{490\,nm}$ was recorded. SEC protein concentrations in the reaction ranged from 15 to 180 µg.

Protein determination—Protein determination followed the method of Bradford (1976). A standard curve in the range of 0–20 µg was constructed using BSA as the standard.

Results

Preparation of semipurified chitinases and chitinases/chitosanases from callus tissue—Four-week old callus tissue was used for enzyme purification because this age of callus had the highest levels of chitinase and chitosanase activities. When the 40–60% desalted AS preparations were subjected to SEC, chitinase and chitosanase activities were detected in those fractions collected between 8.5 to 11 min. Proteins from the crude homogenate, 40–60% and 60–90% AS precipitation steps and fractions active after SEC were examined by SDS-PAGE. The crude callus extract showed two major proteins at M_r 45,000 and 63,000 together with several lower molecular weight proteins. The 43,000 M_r proteins were also present in the 60–90% AS fraction. The 63,000 M_r proteins and most of the lower M_r proteins precipitated at 40–60% AS saturation. The 63,000 M_r and low M_r (<21,500) proteins were removed after SEC. Only the proteins with M_rs of 25,000 to 40,000 remained. This semipurified preparation was used for enzyme characterization.

Enzyme characterization—The effects of pH and temperature on callus tissue chitinase and chitosanase activities in the pooled SEC fraction were determined. Chitinase activity was optimal over a pH range of 3.5 to 5 while chitosanase activity was optimal at pH 5. The reaction temperature optimum for both enzymes was ca. 60°C. Chitinase activity decreased at temperatures above 60°C whereas chitosanase activity began to decrease at temperatures above 70°C. Preincubation of the reaction mixtures at different temperatures for 30 min prior to measurement of enzyme activity revealed that both enzymes retained activity at temperatures up to 60°C.

Studies were conducted to determine if the chitosan source had an effect on chitosanase activity. Table 1 gives the results of these studies and the percentage acetylation for each substrate preparation. The percentage acetylation ranged from 0 to 20%. Citrus chitosanase activity appeared to be optimal with shrimp and krill chitosan with 18–20% acetylation. However, one must be careful in assigning all activity to chitosanases; it is possible that chitinases are contributing to the apparent activity by increasing the concentration of soluble oligosaccharides that contain free amine groups. Chitosanase activity was fairly constant with substrates containing 10.5% or less acetylation which could indicate little or no contribution to observed activity by chitinases.

A determination of endo- and exohydrolase activity with crab chitin and 100% deacetylated shrimp chitosan revealed that the major products were the dimers (i.e. $(GlcNAc)_2$ and $(GlcN)_2$). Minor amounts of monosaccharides were also present. These results indicate that the citrus chitinases and chitosanases are largely endohydrolases.

Attempts were made to determine lysozyme activity in the semipurified SEC preparations. Activity was observed at all of the protein levels tested with the *M. lysodeikticus* suspensions at pH 5. However, there was no activity at any of the protein concentrations used when the assay pH was at 6.24.

Purification of chitinases/chitosanases—A summary

Table 1 The influence of different chitosan samples on the chitosanase activity of sweet orange callus tissue

Chitosan source	% Acetylation	Chitosanase activity (U)
Krill	20.1±0.8	0.90±0.03
Shrimp	18 ±0.5	0.71±0.04
Crab	13.8±1.8	0.40±0.04
Crustaceae	10.5±3.1	0.17±0.02
Crab	5	0.19±0.09
Shrimp	0	0.32±0.03
Crab	0	0.19±0.02

Chitosanase activity was measured as indicated in Material and Methods. Activity and acetylation values are given as the means of four replications and three replications, respectively.

of the purification is given in tabular form in Table 2. The pooled SEC fractions (semipurified preparation) were subjected to HPLC anion exchange chromatography to separate basic from acidic isoforms of chitinase and chitosanase (Fig. 2). Basic proteins were eluted in the void volume after 4.5 min (fraction A0). Acidic chitinases/chitosanases were detected in fractions A1, A2, A3, and A4 eluted after 38, 42, 46, and 50 min, respectively. Fraction A5, which eluted after 57 min, exhibited only chitinase activity. The proteins in each fraction were further purified on either chromatofocusing or anion exchange columns. Two proteins were found in fraction A1 that exhibited chitinase/chitosanase activity and these were further purified to homogeneity (Osswald et al. 1993). Fractions A2 and A3 were subjected to chromatofocusing with a pH range of 6.3 to 4.7 (Fig. 3). Two fractions with elution times of 50 min (A2-CF1) and 54 min (A2-CF2) and eluant pHs of 5.41 and 5.32, respectively, were obtained from A2. Both fractions showed chitinase and chitosanase activities. However, only a single peak was detected at 65 min (A3-CF1) for A3, with an eluant pH of 4.95. A3-CF1 showed only chitinase activity. Fractions A2, A2-CF1, A2-CF2, A3, and A3-CF1 were analyzed by SDS-PAGE. After silver staining, one major (M_r 29,000) and four minor protein bands were visible for fraction A2 (Fig. 4, lane 2). Chromatofocusing A2 yielded a single protein of M_r 29,000 (A2-CF1) by SDS-PAGE (Fig. 4, lane 4); this protein cross-reacted on a western blot with an antibody raised against a basic (class I) potato leaf chitinase (Fig. 4, lane 5). Similar results were found for fraction A2-CF2, except that two minor proteins were visible in addition to the major 29,000 M_r protein (Fig. 4, lane 6). Only the major 29,000 M_r protein cross-reacted with the potato leaf chitinase antibody (Fig. 4, lane 7). Fraction A3 consisted of a major protein (M_r 25,300) together with sev-

Table 2 Purification summary for 'Valencia' callus tissue chitinase and chitinase/chitosanase isoforms

Purification step	Total protein (mg)	Chitinases				Chitosanases			
		Total activity[a]	Specific activity[b]	Purification (-fold)	Yield (%)	Total activity[a]	Specific activity[b]	Purification (-fold)	Yield (%)
(I) Crude Extract[c]	2,180	65,742	30	1	100	179,476	82	1	100
(II) 40–60% AS[c]	1,025	38,364	37.4	1.2	58	89,771	87	1.1	50
(III) SEC[c]	270	17,448	65	2.2	27	34,346	128	1.6	19
(IV) Anion Exchange									
A0	85.4	3,547	42	1.4	5.4	7,842	92	1.1	4.4
A1[c]	0.12	49	404	13.5	0.07	297	2,475	30	0.2
A2	0.53	226	432	14.4	0.34	783	1,497	18.3	0.4
A3	0.57	128	224	7.5	0.19	398	696	8.5	0.2
A4	6.86	638	93	3.1	0.9	855	125	1.5	0.5
A5	2.46	204	83	2.8	0.3	na	—	—	—
(V) Chromatofocusing									
A1-CF1[c]	nd	14.6	nd	nd	0.02	55.1	nd	nd	0.03
A1-CF2[c]	nd	20	nd	nd	0.03	58	nd	nd	0.03
A2-CF1	nd	31	nd	nd	0.04	266	nd	nd	0.15
A2-CF2	nd	29	nd	nd	0.04	178	nd	nd	0.1
A3-CF1	nd	51	nd	nd	0.08	na	—	—	—
A5-CF1	nd	9.7	nd	nd	0.01	na	—	—	—
A5-CF2	nd	12.2	nd	nd	0.02	na	—	—	—
A5-CF3	nd	15.3	nd	nd	0.02	na	—	—	—
A5-CF4	nd	9.8	nd	nd	0.01	na	—	—	—
(VI) Anion Exchange									
A4-AN1	0.99	255	257	8.56	0.39	na	—	—	—
A4-AN2	0.55	116	211	7.03	0.18	na	—	—	—

The Roman numerals indicate the successive steps of purification according to Fig. 1.

[a] Chitinase or chitosanase total activity=Units.

[b] Chitinase or chitosanase specific activity=Units per mg protein.

[c] Data from Osswald et al. 1993.

na, no activity detected; nd, not determined due to the interference of ampholytes from the chromatofocusing step.

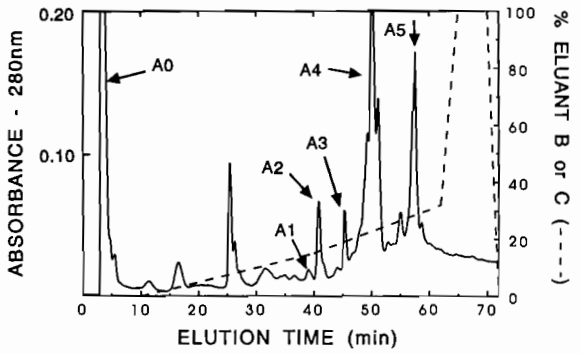

Fig. 2 Elution pattern of chitinases and chitosanases on an anion exchange column. The pooled, concentrated fractions from the SEC step were loaded on the anion exchange column as described in Materials and Methods. The proteins were eluted using a two-step linear gradient as described by Osswald et al. (1993). Absorbance at 280 nm; ---, Eluant buffers B and C.

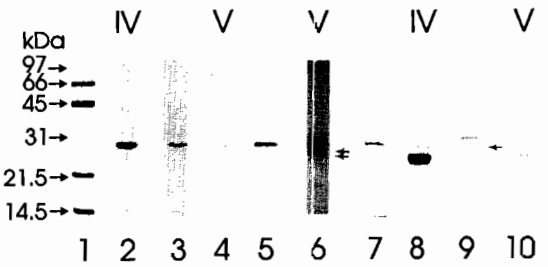

Fig. 4 SDS-PAGE and immunoblots of protein fractions A2, A2-CF1, A2-CF2, A3, and A3-CF1. Roman numerals indicate the successive steps of purification according to Figure 1. Lane 1, molecular weight markers; lane 2, SDS-PAGE of A2; lane 3, western blot of A2; lane 4, SDS-PAGE of A2-CF1; lane 5, western blot of A2-CF1; lane 6, SDS-PAGE of A2-CF2; lane 7, western blot of A2-CF2; lane 8, SDS-PAGE of A3; lane 9, western blot of A3; lane 10, SDS-PAGE of A3-CF1. Lane 2 was loaded with 5, lane 8 with 8 μg protein. All other lanes were loaded with 2 μg protein.

eral proteins with higher and lower molecular weights (Fig. 4, lane 8). Only two of these (M_r 28,000 and 30,900) cross-reacted with the potato chitinase antiserum used above (Fig. 4, lane 9). A single M_r 26,200 protein (A3-CF1) was found for fraction A3 after chromatofocusing and SDS-PAGE (Fig. 4, lane 10). This protein possessed chitinase activity only (Table 2) and did not cross-react with the antibody (data not shown). These results indicate that the two proteins that cross-reacted with the potato chitinase antiserum were lost during chromatofocusing. Fraction A4 was applied to an anion exchange column a second time using a slightly modified elution buffer system. These conditions resulted in a baseline separation of two major proteins, A4-AN1 and A4-AN2 (Fig. 5). These two protein fractions possessed only chitinase activity (Table 2) and appeared as single bands on SDS-PAGE (Fig. 6, lanes 4, 6). Both chitinases cross-reacted with the potato leaf chitinase antibody and had M_rs calculated to be 30,600 (Fig. 6, lanes 5, 7). Fraction A5 (SDS-PAGE shown in Fig. 6, lane 8) was further separated on a chromatofocusing column within a pH range of 6.3 to 3.3. Six fractions were found (chromato-

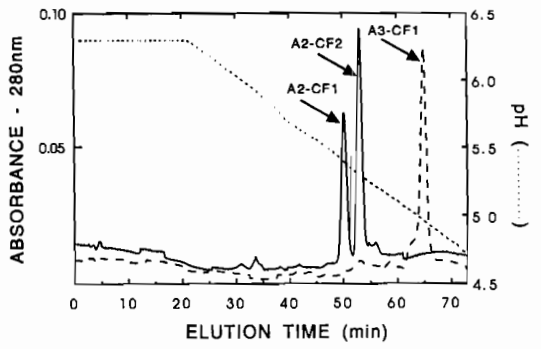

Fig. 3 Elution patterns for A2 and A3 chitinases and chitinase/chitosanases from a chromatofocusing column. Proteins of fractions A2 and A3 were concentrated, dialyzed against 25 mM bis-Tris buffer, pH 6.3 and separated at 1 ml per min as described in Materials and Methods. Elution profiles represent separate injections of fractions A2 and A3. —, (A2); ---, (A3); —, Absorbance at 280 nm; ----, Eluant-pH.

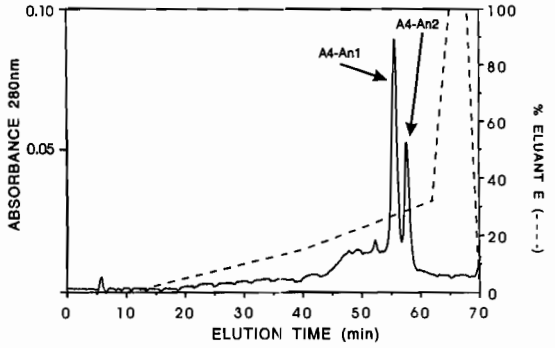

Fig. 5 Elution pattern of chitinases and chitosanases of fraction A4 on an anion exchange column. Fraction A4 proteins were concentrated, dialyzed against buffer D, and loaded on the anion exchange column equilibrated with buffer D. The proteins were eluted using a two-step linear gradient (0–15% E, 10–40 min; 15–32% E, 40–62 min). The column was washed after each run with 100% buffer D for 5 min. —, Absorbance at 280 nm; ---, Eluant buffer E.

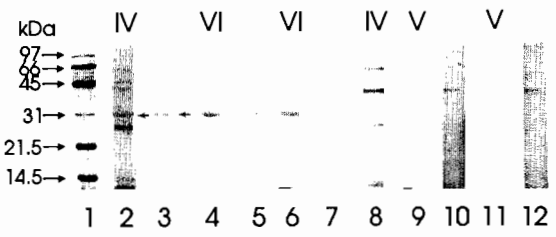

Fig. 6 SDS-PAGE and immunoblots of protein fractions A4, A4-AN1, A4-AN2, A5, A5-CF3, and A5-CF4. The Roman numerals indicate the successive steps of purification according to Figure 1. Lane 1, molecular weight markers; lane 2, SDS-PAGE of A4; lane 3, western blot of A4; lane 4, SDS-PAGE of A4-AN1; lane 5, western blot of A4-AN1; lane 6, SDS-PAGE of A4-AN2; lane 7, western blot of A4-AN2; lane 8, SDS-PAGE of A5; lane 9, SDS-PAGE of A5-CF3; lane 10, western blot of A5-CF3; lane 11, SDS-PAGE of A5-CF4; and lane 12, western blot of A5-CF4. Lanes 2 and 8 were loaded with 8 μg protein. The other lanes were loaded with 2 μg protein.

gram not shown), but, only four (A5-CF1, A5-CF2, A5-CF3, A5-CF4) exhibited chitinase activity (Table 2). Each fraction was concentrated, analyzed on SDS-PAGE, and probed with potato leaf chitinase antiserum (Fig. 6). After silver staining, only fractions A5-CF3 and A5-CF4 were homogenous on SDS-PAGE (Fig. 6, lanes 9, 11). Both proteins cross-reacted slightly with the antibody mentioned above and were characterized by a M_r of 37,400 (Fig. 6, lanes 10, 12).

N-terminal sequencing—Partial N-terminal sequences obtained for A2-CF1, A2-CF2, and A4-A1 are shown in Table 3 along with some known sequences for class I and class III chitinases for comparison purposes. Several other proteins were submitted for sequencing but unidentified problems (possibly blockage or chemical interference) prevented sequencing. The sequences from A2-CF1 and A2-CF2 were identical and have homology with the class III chitinase from *Arabidopsis*. The N-terminal sequence from A4-AN1 exhibits homology with the class I chitinase from *Arabidopsis* but lacks the cysteine rich N-terminal region which distinguishes class I chitinases (Shinshi et al. 1990). Consequently, by definition A4-AN1 is a class II chitinase.

Discussion

Table 4 summarizes the characteristics of the chitinases isolated from sweet orange callus tissue. Eleven chitinase isoforms were obtained. Four of the 11 enzymes exhibited chitinase and chitosanase activities and the remainder had chitinase activity only. No acidic isoform was obtained that degraded only chitosan as has been reported by Grenier and Asselin (1990) and Ouakfaoui and Asselin (1992a, b). These researchers described acidic and basic chitosanase isoforms with M_rs between 10,000 and 23,000 in roots, leaves, fruits, and flowers of dicots and monocots using activity-staining techniques on gels with glycolchitosan as the substrate. Citrus proteins in the 10,000 to 23,000 M_r range did not have any detectable chitosanase activity (data not shown) and were eliminated as a result of the SEC purification step. We have also confirmed the presence of chitinases and chitinase/chitosanases in roots, stems, leaves and flowers of citrus (unreported data). Chitinase/chitosanases have been reported previously. For example, a constitutive hydrolase with dual chitinase and chitosanase activity was described by Pegg and Young (1982) in tomato stems that had a pI of 8.5 and an M_r between 27,000 and 30,000 and recently Watanabe et al. (1992) reported two bacterial chitinases which also showed chitosanase activity.

The results reported in Table 1 indicate that the citrus callus chitinases/chitosanases were active with completely deacetylated chitosan and that activity decreased with a decreasing percentage acetylation of chitosan. This is in general agreement with prior results obtained from similar studies with *Streptomyces griseus* chitosanases (Osswald et al. 1992). The data obtained with 100% deacetylated chitosans provide conclusive evidence that true chitosanases (predominantly endohydrolases) are present in *Citrus*. Little is known about the specificity of purified plant chitinases/chitosanases and chitosanases with chitosans that have been acetylated to different degrees. Grenier and Asselin (1990) commented that more than 5–6% acetyla-

Table 3 Comparison of citrus chitinase N-terminal sequences with known sequences from *Arabidopsis thaliana* (Samac et al. 1990), *Cucumis sativis* (Métraux et al. 1989), *Parthenocissus quinquifolia* (Bernasconi et al. 1987), *Hevea brasiliensis* (Jekel et al. 1991), and *Cicer arietinum* (Vogelsang and Barz 1993)

Class I & II	
Arabidopsis	E Q C G R Q A G G A L C P N G L C C S E F G W C G N T E P Y C K Q P G C Q S Q C T P G G T P P G P T G D L S G I I S S S Q F D D M L K H R N D A A C P A R G F Y T Y N A F I T A A K S F P G F G T T G
A4-AN1	. . . . . * E * Q K * * F R E M * * * L * E Y * * * E R * * * * * * * * * D * * * E * * Q A * * * * * N S *
Class III	
Arabidopsis	G G I A I Y W G Q N G N E G N L S A T C A T G R Y A Y V N V
Cucumis	A * * * * * * * * * * * * S * A S * * * * * N * E F * * I
Hevea	* * * * * * * * * * * * * * T * T Q * * S * R E * S * * I
Parthenocissus	* * * * * * * * * * * * * * T * T Q * * S * * K * S * * I
Cicer	A * * * V * * * * * * * * * S * Q D A * N * N N * Q F * * I
Citrus	
A2-CF1	* V * S V * * * * * * * * * S * A D A X S S * N * X I * K I
A2-CF2	* V * S V * * * * * * * * * S * A D A X S S * N * X I * K I

Residues identical with those from *Arabidopsis* are marked with asterisks. Undetermined residues are marked with Xs.

Table 4 Summary table of acidic chitinases/chitosanases from 'Valencia' callus tissue

Hydrolase No.	M_r (10^3)	pI [a]	Crossreact [b]	Homogenous	Chitinase activity	Chitosanase activity
A1-CF1 [c]	30.5	5.4	yes	yes	yes	yes
A1-CF2 [c]	30.5	5.29	yes	yes	yes	yes
A2-CF1	29.1	5.41	yes	yes	yes	yes
A2-CF2	29.1	5.32	yes	no	yes	yes
A3-CF1	26.2	4.95	no	yes	yes	no
A4-AN1	30.6	4.56	yes	yes	yes	no
A4-AN2	30.6	4.5	yes	yes	yes	no
A5-CF1	31.9	4.96	yes	no	yes	no
A5-CF2	31.9	4.81	yes	no	yes	no
A5-CF3	37.4	4.76	yes	yes	yes	no
A5-CF4	37.4	4.62	yes	yes	yes	no

[a] Determined by chromatofocusing.

[b] Cross-reaction determined in western blots using a basic class I potato leaf chitinase antibody.

[c] See ref. Osswald et al. (1993).

tion of chitosan would not allow the differentiation of hydrolysis of glycol chitosan from glycol chitin in activity stains with plant PR proteins and suggested that there may be enzymes present that have the ability to hydrolyze both substrates. It should not be surprising that chitosanases are active with partially acetylated chitosans as chitosans isolated from fungal sources are partially *N*-acetylated (Wessels and Sietsma 1981).

The M_rs of the citrus chitinases we report (i.e., M_rs of 26,000–37,000; Table 4) are within the size range of chitinases reported previously (Carr and Klessig 1989, Bol et al. 1990, Flach 1992). The pIs of the callus tissue hydrolases as determined by chromatofocusing were between 4.5 and 5.4. The pIs for the acidic chitinases from citrus are similar to those for acidic chickpea (Vogelsang and Barz 1993) and other plant chitinases (Carr and Klessig 1989, Bol et al. 1990, Flach 1992).

The sequencing data for three of the enzymes (A2-CF1, A2-CF2, A4-AN1; Table 3) indicate that the isoforms are class II and III chitinases. All of the sequenced citrus enzymes cross-reacted with the class I basic potato leaf chitinase antibody (Table 4). Cross-reactivity suggests that the enzymes are antigenically similar and possess homology to potato leaf chitinases. It is understandable that the class II chitinase (A4-AN1) would cross-react with the antibody as there is some sequence homology to the class I enzymes. We are at a loss at this time to adequately explain the cross-reaction with the class III chitinases/chitosanases. We have run western blots employing a potato leaf β-1,3-glucanase antibody obtained from the same laboratory and observed no cross-reaction with the antigen yielding a positive reaction with the potato leaf class I chitinase antibody; this would eliminate the possibility that there could be a procedural error. A possible explanation would be that there may be some sequence homology that has not yet been revealed by the N-terminal sequencing. Perhaps some insight will be provided when additional sequencing data become available for the citrus chitinases/chitosanases. Finally, we cannot rule out the possibility that there is either a class I or II chitinase or other antibody cross-reacting protein contaminant that could not be separated with the isolation techniques used.

Some of the citrus chitinase/chitosanase isoforms are very similar based on the pI values, apparent M_rs, and the partial N-terminal sequences for A2-CF1 and A2-CF2. Until either more amino acid or gene sequencing data are available for these proteins we cannot rule out the possibility that some of them are the same and vary only by a few amino acids as a result of post-translational modifications. At this point we are confident that two chitinase classes are present. On the basis of M_rs there may be 8 different acidic isoforms.

Very little information has been published on the PR or PR-like proteins from citrus. Gavish et al. (1991) have reported that antibodies raised against tobacco chitinases and β-1,3-glucanases cross-reacted with extracellular proteins isolated from sour orange (*Citrus aurantium*) nucellar-derived cell cultures. Gentile et al. (1993) correlated high levels of chitinase and β-1,3-glucanase activities in 'Tarocco' orange and resistant 'Femminello' lemon nucellar callus cell lines with inhibition of co-cultured mal secco (*Phoma tracheiphila*) colonies. To our knowledge, we are the first to have purified and characterized hydrolases from citrus which degraded chitin and chitosan (Osswald et al. 1993). Chitinases that are also chitosanases and lysozymes could be very effective in disrupting host-pathogen and host-insect interactions since they would be active against a wider variety of plant pests. Also, oligosac-

charides produced as a result of chitinase/chitosanase/lysozyme action on fungal pathogens may act as elicitors to induce or trigger host defense reactions. From the standpoint of energy conservation in a biological system it is much more efficient to have multifunctional enzymes.

We thank Dr. E. Kombrink for the gift of the potato leaf chitinase antiserum and Drs. S. Aiba, D. Koga and the Katakura Chikkarin Co., Ltd., for the chitosan samples. We are indebted to Drs. A. Asselin, T. Boller, M. Legrand, R. Sonoda and G. McCollum for critically reading the manuscript and providing advice. We are grateful to Nancy Denslow and the Interdisciplinary Center for Biotechnology Research. We also thank Mike Burkhart and Troy Pierce for assistance with the assays. Dr. W. Osswald was the recipient of an Alexander von Humboldt Feodor Lynen Postdoctoral Fellowship and an Agricultural Research Service Administrator's Research Associate Award during the conduct of this research.

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(Received February 17, 1994; Accepted May 7, 1994)